DEVELOPMENTAL MORPHOLOGY OF ASCOMYCETES

IX. *CALONECTRIA RIGIDIUSCULA*

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SUMMARY. – Study of the developmental morphology of an homothallic strain of *Calonectria rigidiuscula* (Berb. & Br.) Sacc. and its anamorph *Fusarium decemcellulare*.

RÉSUMÉ. – Étude de l'organogenèse d'une souche homothallique de *Calonectria rigidiuscula* (Berk. & Br.) Sacc. et son anamorphe *Fusarium decemcellulare*.

This paper is the ninth in a series on the developmental morphology of Ascomycetes and deals with *Calonectria rigidiuscula* (Berk. & Br.) Sacc. The observations presented here are based on a study of a homothallic strain of *C. rigidiuscula*, isolated from dead twigs of cocoa, collected at Jodupala, Coorg District, Karnataka State, India.

Calonectria rigidiuscula was originally described as *Nectria rigidiuscula* by BERKELEY and BROOME in 1873 based on their collection of a fungus from Ceylon («Fungi of Ceylon, N° 1024»). BERKELEY and BROOME gave the following diagnosis :

«Caespitosa; perithiciis ovatis pallide coccineis vix collabentibus; sporidiis submetulaeformibus quadrinucleatis, demum 3-septatis (No 173C). On bark».

Since the ascospores were 3-septate (and not 1-septate), SACCARDO (1878) transferred *Nectria rigidiuscula* to *Calonectria* de Notaris as *C. rigidiuscula* (Berk. & Br.) Sacc. De NOTARIS (1867) erected the genus *Calonectria* from nectriaceous fungi possessing fusoid, large, 2-multiseptate, hyaline ascospores, with *C. daldiniana* de Not. as the type; he wrote : «Si distingue questo tipo dalle *Nectria*, per gli sporidii fusoidi, allungati, pluriloculari» (de NOTARIS, 1867, p. 477). While transferring *Nectria rigidiuscula* to *Calonectria*, SACCARDO

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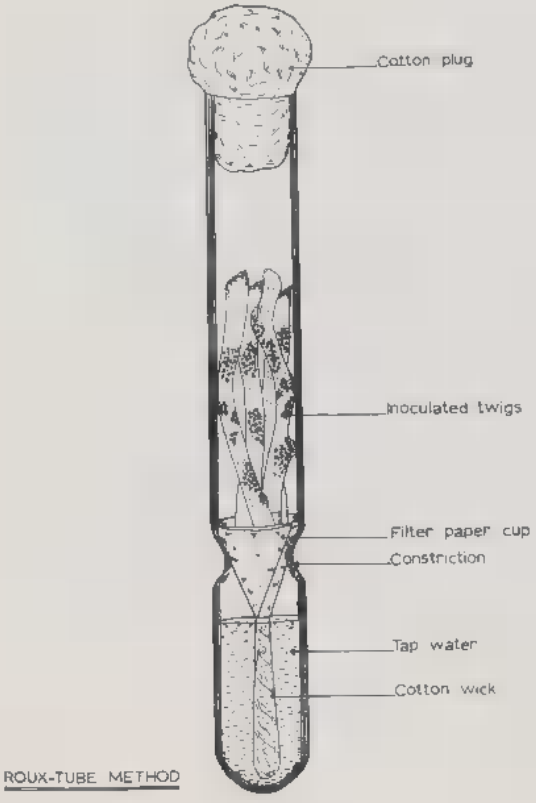
(1878) noted the incompleteness of BERKELEY and BROOME's description of the fungus, but as his list was only a compilation, he merely reproduced the original diagnosis which did not give information on asci. In his revision of Ceylon Hypocreales, PETCH (1916) stated that BERKELEY and BROOME's description of the fungus was incomplete and incorrect as to colour of the perithecia. BOOTH (1960) redescribed the fungus from a study of the type specimen of *Nectria rigidiuscula*.

The conidial state of *Calonectria rigidiuscula* is *Fusarium decemcellulare* Brick. WOLLENWEBER (1926) established the connection between *Calonectria rigidiuscula* and *Fusarium decemcellulare*.

REICHLE & SNYDER (1964) recognized both homothallic and heterothallic strains in *Calonectria rigidiuscula*. Homothallic strains invariably formed 4-spored asci, whereas heterothallic strains produced 2-8 spored asci. The fungus is mainly tropical in distribution (BOOTH, 1971). It occurs widely as a saprophyte and is also known to cause dieback of twigs of many plant species, including cocoa. BRUNT & WARTON (1962) reported that the fungus causes galls in cocoa. According to FORD et al. (1967) only isolates of heterothallic strains induced gall in cocoa seedlings. In India, this fungus was first reported by SUBBA RAO (1938).

METHODS



ROUX-TUBE METHOD

To obtain perithecia, single ascospore isolates were inoculated on sterilized twigs of cocoa in Roux tubes. Perithecia developed and matured in about eight weeks. The Roux-tube (Fig. 1) method is as follows :

Roux-tubes (Borosil Glass Works Ltd) of Corning brand (30 cm long and 3 cm diam.) were used as containers. Each tube was filled with tap water up to the constriction and a filter paper cup was kept above the latter. A cotton-wick inserted through the cone end of the filter paper connected the water to the filter paper and thus kept the space above the filter paper moist. Dead twigs of cocoa, the one on which the fungus was originally isolated, were cut into pieces each about

12 cm long. The twigs were pruned to remove the side branches. Surface debris was washed off with a brush in running water. The twigs were placed above the filter paper in the Roux-tubes and sterilized twice under 20 lb/cm^2 pressure for 20 min. with an interval of 48 hr. The stem pieces were then inoculated with the fungus and incubated under continuous daylight of 200 ± 25 ft. candles at a temperature of 22-25°C.

For studying the various stages in the development of the anamorph and teleomorph, methods described earlier (SUBRAMANIAN & BHAT, 1978) were followed.

DESCRIPTION OF THE FUNGUS

Mycelium on the twig not visible on the surface, subcortical, forming pseudoparenchymatous stromata in the cortical region. Stromata 1-3 mm diam., occasionally extending up to the surface, composed of angular, thin-walled, hyaline cells 9.0-12.0 μm, in diam. Perithecia (Fig. 53; Plate I, 1) superficial, solitary or crowded in groups of 2-8, globose to subglobose, cream to whitish yellow in colour, fleshy, warty, not undergoing lateral collapse when dry, globose perithecia 240-360 (310) μm in diam.; subglobose perithecia 220-300 (270) x 240-380 (320) μm. Perithecial wall (Fig. 52; Plate II, g) pseudoparenchymatous, warted in the upper half, translucent, 45-75 μm wide, with 2 distinct regions : an outer and an inner. Outer region with 4-5 tiers of cells, 20-35 μm thick, cells spherical to angular, slightly thick-walled, hyaline, 15-20 μm in diam. Inner region with 3-4 tiers of cells, 15-20 μm thick; cells flattened, narrow, elongated, compactly arranged, 11-14 x 2.5-3.5 μm. Warts (Plate II, f) pseudoparenchymatous, 20-50 μm high, with cells similar to those of the outer region of the perithecial wall. Perithecial papilla subacute, composed of parallely arranged, cylindrical, septate, unbranched hyphae with rounded ends, protruding through the outer region of the perithecial wall. Ostiolar canal periphysate; periphyses subcylindrical, slender, 15-18 x 1.8 μm with rounded and subacute tips (Fig. 54).

Asci clavate, short-stalked, smoothly rounded at the apex, without apical apparatus, unitunicate, thin-walled, generally 4-spored, 70-110 (85) x 10-14 (13.8) μm (Fig. 68; Plate II, h); 8-spored asci present in heterothallic strains, larger than 4-spored asci, clavate, distinctly pedicellate, broadly rounded at the apex, without apical apparatus, 95-130 (105) x 12.5-16.0 (14.5) μm (Fig. 71; Plate II, j). Ascospores (Fig. 69; Plate II, i) similar in 4-spored and 8-spored asci, ellipsoidal to reniform, thick-walled, 3-septate, with uninucleate cells (Fig. 63), hyaline, slightly constricted at the septa, 20-30 (24.5) x 7.0-10.5 μm; ascospores uniseriate with overlapping ends in 4-spored asci and biseriate above and uniseriate below in 8-spored asci.

CULTURAL CHARACTERS

Ascospores germinating within 14 hrs on potato dextrose agar, corn husk agar and in distilled water, producing one germ tube from each cell (Fig. 70).

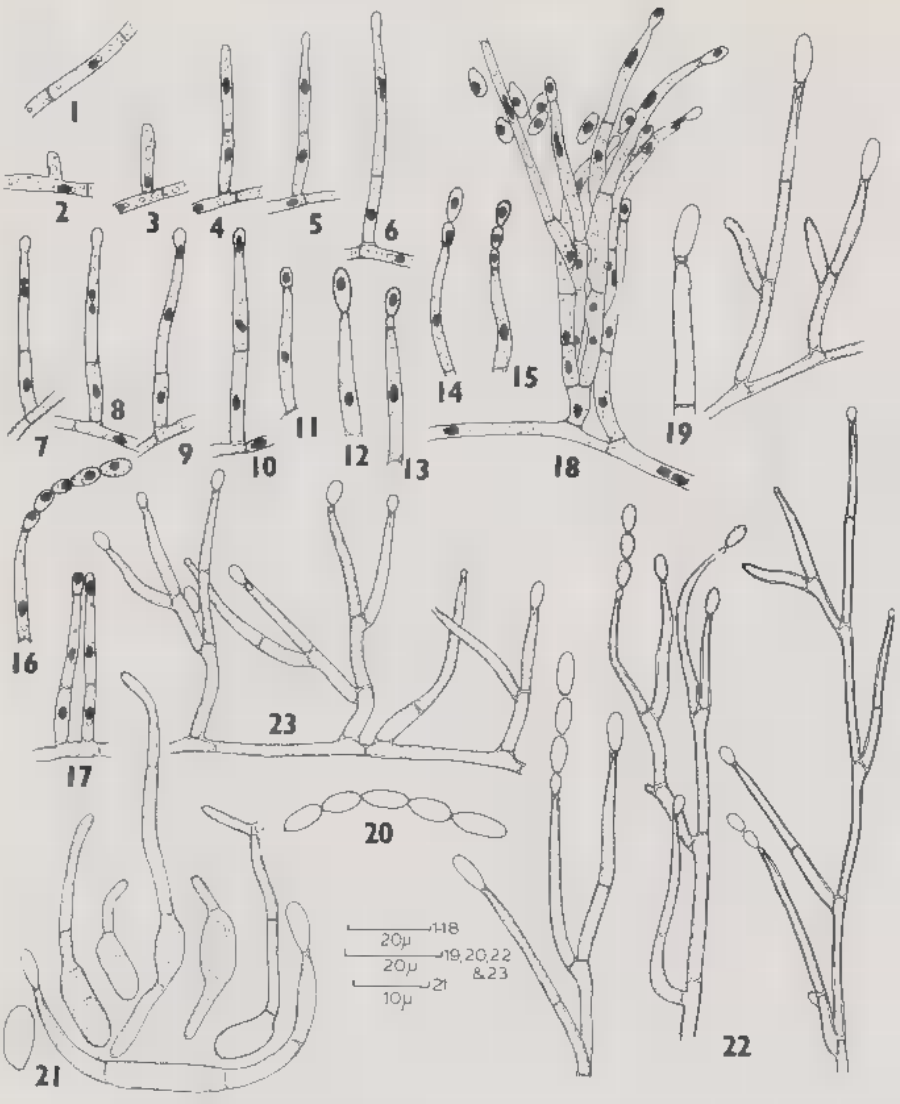


Fig. 1-23 : *Calonectria rigidiuscula*. — 1 : portion of a vegetative hypha; 2-12 : stages in the development of phialide and first microconidium; 13-16 : development of second and later conidia; 17, 18, 22, 23 : arrangement of phialides on conidiophores; 19 : a phialide. Note the cupulate collarette; 20 : a pseudochain of microconidia; 21 : germination of microconidia.

Colonies on potato dextrose agar attaining a diam. of 3.2-3.8 cm in 10 days, floccose, with surface initially white to pinkish and later becoming rose-coloured, with reverse initially rose-coloured and later becoming brick-red: on corn

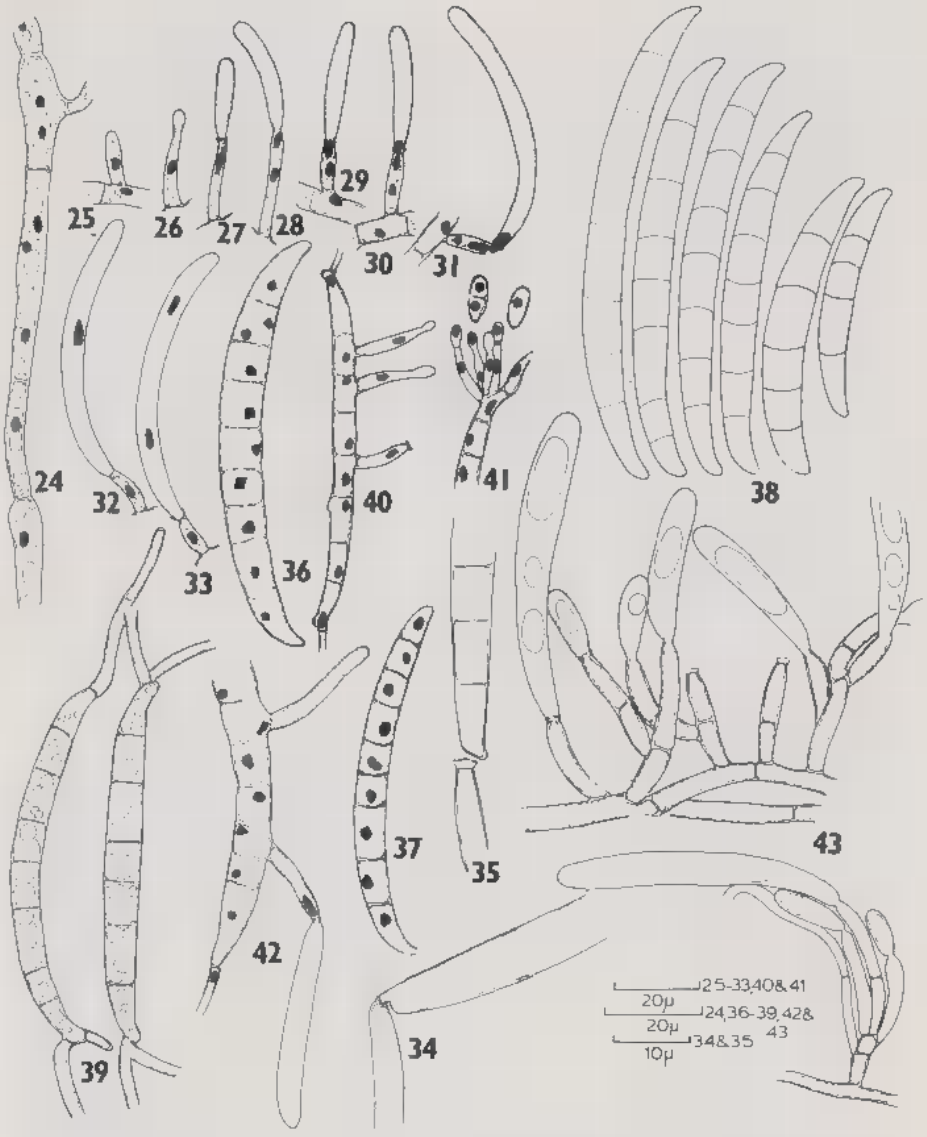


Fig. 24-43 : *Calonectria rigidiuscula* — 24 : portion of a vegetative hypha; 25-33 : stages in the development of phialide and macroconidium; 34-35 : portion of phialides. Note in 35, the cupulate collarette; 36-38 : macroconidia. Note in 36 and 37, the uninucleate cells; 39 : germinating macroconidia; 40-42 : germ tubes functioning as phialides. Note in 41, microconidia produced from phialides and in 42, macroconidia produced on phialides; 43 : arrangement of phialides on conidiophores.

husk agar attaining a diameter of 2.5-3.0 cm in 10 days, adpressed, slimy, with surface rose-coloured, with reverse brick red. Mycelium fast-growing, composed of septate, branched, hyaline hyphae 2.5-4.0 µm wide, producing conidiophores initially in the centre of the colony and later all over the surface. Conidiophores developing laterally on the hyphae, straight or bent, up to 200 µm long, branched or unbranched, with uninucleate cells and terminating in phialides (Fig. 22, 23, 43).

Phialides cylindrical to subulate, slightly wide at the base, narrow towards the tip, with a collarette, uninucleate, 30-42 (35.5) x 4.5 µm (Fig. 17-19, 35; Plate I, d). Conidia of two types : microconidia and macroconidia. Microconidia (Fig. 16, 20; Plate I, c, e, h) formed singly and in basipetal succession, solitary, remaining in pseudochains, 1-celled, oval to cylindrical, rounded at the apex, narrowed at the base, hyaline, uninucleate, 10.0-14.5 (12.5) x 3.0-4.5 µm. Macroconidia (Fig. 36-38; Plate I, b, i) broadly falcate, distinctly dorsi-ventral, 7-10-septate, with a distinct foot-cell at the base, apically beaked, thick-walled, hyaline, 55-130 (110) x 6.0-10.5 µm; cells uninucleate and with dense cytoplasm. In aged cultures phialides aggregating and forming sporodochia (Plate I, a), producing conidia in cream coloured slimy mass. Each cell of the macroconidia capable of producing germ tubes, usually germ tubes arising from terminal cells (Fig. 39; Plate I, j).

DEVELOPMENT OF THE ANAMORPH

Conidia develop in 4-5 days in slide culture. Conidiophores arise laterally or terminally on the vegetative hyphae (Fig. 18). Phialides are borne on conidiophores or directly on the vegetative hyphae (Fig. 2-4 Plate I, c, d). Immediately below the basal septum of the phialide, one or two lateral protuberances may grow out, with each one becoming a phialide. Phialides are widest at the base, tapering towards the neck and with a cupulate collarette at the tip.

The development of the microconidium is as follows. The conidium initial arises as a small bud at the tip of a phialide (Fig. 5). With further development, the bud increases in size. In the meantime, the uniformly stained spherical nucleus of the phialide elongates parallel to the long axis of the phialide (Fig. 6) and divides into two daughter nuclei (Fig. 7, 8; Plate I, e, f). One of the two daughter nuclei migrates into the conidium initial above (Fig. 9, 10; Plate I, g). By the time the nucleus enters conidium initial, the conidium almost attains its full size. With the formation of a septum, the slender cytoplasmic connection between the phialide and the conidium is disrupted (Fig. 11). The wall of the conidium initial breaks during the final stages of development presumably at a weak point located just above the narrow neck of the phialide (Fig. 12). Often an irregular fracture of the wall takes place leaving behind a cupulate collarette (Fig. 19; Plate I, d). As the first conidium is cut off, a second conidium initial appears in the open end of the phialide and the first conidium is pushed up (Fig. 13, 14). The process is repeated and a succession of conidia are produced. The conidia produced from each phialide remain in a linear series (pseudochains)

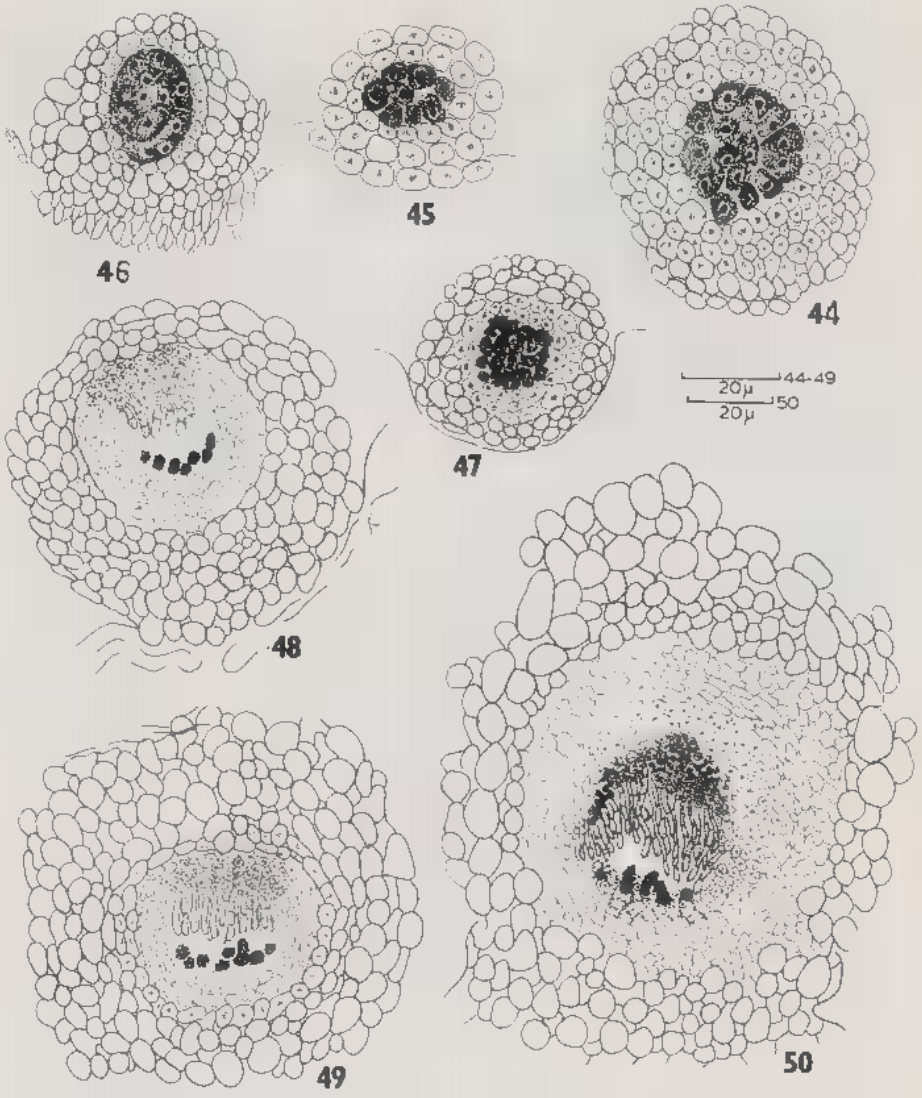


Fig. 44-50 : *Calonectria rigidiuscula* (Contd.) – 44-47 : sections of stroma showing stages in the development of the ascogonium. Note in 44 and 47, the uninucleate condition of the ascogonial cells and in 45 and 46, uni- or binucleate condition. Note also in 46 and 47, ascogonial cells surrounded by one to two layers of deeply staining thin-walled cells; 48-50 : sections through young perithecial centra showing stages in the formation of centrum cavity, development of apical paraphyses and differentiation of perithecial wall. Note the ascogonial cells lying at the base of the cavity.

due to slime (Fig. 15, 16, 20; Plate 1, c, h). Occasionally, the conidia slime down into a gloeoid mass. Conidia germinate producing one to two germ tubes (Fig. 21).

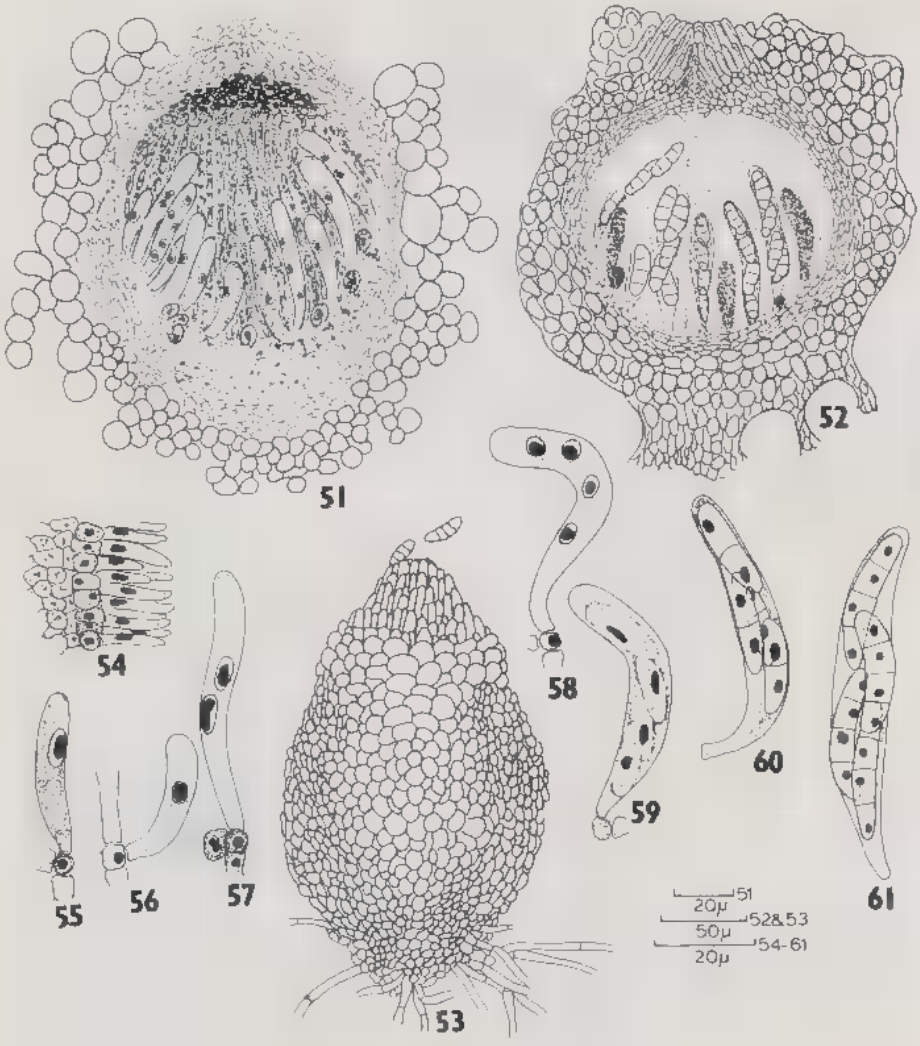


Fig. 51-61 : *Calonectria rigidiuscula* (Contd.) — 51 : section through a young perithecium; 52 : section through a mature perithecium; 53 : a mature perithecium (whole mount). Note the warty outer surface; 54 : periphyses; 55-61 : stages in the development of ascus and ascospores.

The development of the macroconidium is as follows. The phialide is uninucleate (Fig. 25). During the formation of a macroconidium, a small protuberance arises at the tip of the phialide (Fig. 26). The protuberance elongates and increases in size. The nucleus of the phialide elongates and divides (Fig. 27-29) and one daughter nucleus migrates into the developing conidium (Fig. 30,

31). The appearance of a minute collarette even in the early stages of development of the macroconidium suggests that the rupture of the outer wall of the phialide takes place presumably at the extreme tip of the phialide. The edge of the collarette in most cases is uneven (Fig. 35, 43). The nucleus within the conidium initial now divides repeatedly and finally a multiseptate conidium with uninucleate cells results (Fig. 32, 33, 36; Plate I, i). When placed in distilled water on a slide, conidia germinate after 12 hr. The germ tubes sometimes function as phialides and produce microconidia or macroconidia (Fig. 40-42; Plate I, j).

DEVELOPMENT OF THE TELEOMORPH

Perithecial development is initiated within a stroma which, as already mentioned, develops within the cortex of the substrate. The first indication of the development of the perithecium is the formation of an ascogonium embedded in a pseudoparenchymatous stroma (Fig. 44; Plate II, a). The mature ascogonium is a coiled, septate structure and is composed of about 7-8 uni- or binucleate cells with dense cytoplasm (Fig. 46), the ascogonial cells being uninucleate when young (Fig. 45). No hyphal fusions or anastomoses are noticed and the binucleate condition of the ascogonial cells is presumably a product of mitotic division of nuclei. The ascogonial cells divide further and form a mass of compactly arranged cells. The ascogonium is soon surrounded by 1-2 layers of polygonal cells which stain deeply compared to the cells of the stroma forming, as it were, an envelope (Fig. 47). The envelope increases in size and soon becomes several cells thick and this becomes the wall of the perithecial initial. In the meantime, the young perithecium starts projecting out of the stromal surface (Fig. 48; Plate II, b).

In further development, a cavity develops around the ascogonial elements presumably due to disintegration of cells (Fig. 48, 49). The cells of the perithecial wall in the periphery become thick-walled, pseudoparenchymatous and aggregate into conical warts in the upper half (Fig. 50). As the differentiation of the wall takes place, at the morphological apex of the young perithecium, the cells of the inner region of the perithecial wall become «meristematic» and give rise to intensively staining, small, angular cells. This «meristem» produces a mass of downward growing slender filaments, the apical paraphyses which contain thin-walled, narrow, uninucleate cells (Fig. 48-50; Plate II, c). Initially, the filamentous nature of the apical paraphyses is very evident. As they grow downwards, the cells of the apical paraphyses are swelled laterally forming, as it were, a compact mass of pseudoparenchymatous tissue (Plate II, d). The cells at the base of the perithecial cavity also undergo division and form a mass of globose cells (Fig. 51).

When the perithecium becomes mature, the apical paraphyses regain their filamentous nature (Fig. 51; Plate II, e, f). The asci grow up interspersed with the apical paraphyses forming a kind of hymenium along the bottom and in

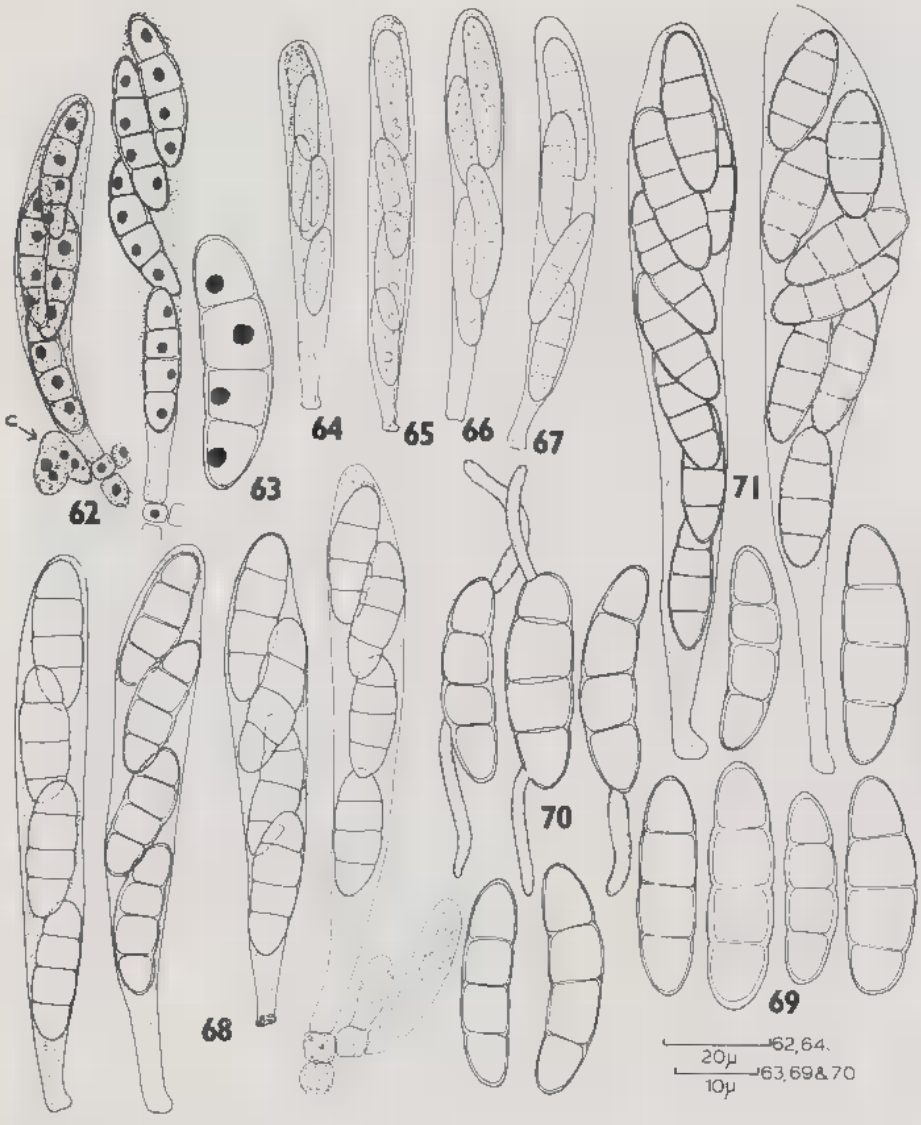


Fig. 62-71 : *Calonectria rigidiuscula* (Contd) – 62 : mature asci. Note a four-nucleate crozier at the base of one of the asci (arrow), c - crozier; 63 : mature ascospore with uninucleate cells; 64-67 : stage in the development of ascus and ascospore; 68 : four-spored asci; 69 : ascospores; 70 : germinating ascospores; 71 : eight-spored asci (from heterothallic strain).

the sides of the perithecial cavity. In mature perithecium, the apical paraphyses disintegrate (Fig. 52).

While the apical paraphyses continued to grow downwards, the cells of the inner region of the perithecial wall at the apex of the perithecium grow upwards and develop an ostiolar neck. The cells constituting ostiolar neck become thick-walled and are in the form of unbranched hyphae with rounded tips. By dissolution of cells in its core, a narrow canal develops. The ostiolar canal extends from the centrum cavity to the exterior. The cells lining the ostiolar canal produce slender, uninucleate cells, the periphyses.

DEVELOPMENT OF ASCI

The ascogonial cells are multinucleate. Each ascogonial cell puts out 2-3 short, thick, ascogenous hyphae which bend over at the tip and form croziers (Fig. 62). Two nuclei move into an ascogenous hypha and the latter is cut off from the ascogonial cell by a septum at the base, the two nuclei divide simultaneously to form four nuclei. Two septa are formed in the crozier separating the latter into a uninucleate basal cell, a binucleate median cell and a uninucleate tip cell. The median cell elongates and the fusion nucleus migrates to the middle of the ascus initial (Fig. 55) and here it undergoes meiosis. In the early stages of meiotic division, seven bivalents are observed (Fig. 56). The first meiotic division results in two nuclei (Fig. 57) and the second in four haploid nuclei (Fig. 58). Cleavage of the cytoplasm takes place in such a way as to produce elliptical masses around each nucleus (Fig. 59). Cell wall is laid down around each elliptical mass to produce an incipient ascospore (Fig. 64-66). Each ascospore nucleus now undergoes a mitotic division, followed by a septum formation across the middle (Fig. 60). Each nucleus of the two-celled ascospore again undergoes a mitotic division and is followed by a septum formation. Thus, eventually, a four-celled ascospore, characteristic of the fungus, is resulted (Fig. 61, 62, 67).

Often the basal cell and the tip cell of a crozier may fuse and give rise to another ascus.

DISCUSSION

The anamorph of *Calonectria rigidiuscula* is a *Fusarium*, the microconidia being produced in long pseudochains. The ascogonium is a several-celled, coiled structure which appears within a preformed stroma and the ascogonial cells originally uninucleate become multinucleate. The perithecial centrum is of the typical *Nectria*-type (LUTTRELL, 1951) with apical paraphyses. The asci have no apical apparatus. Ascospores are multiseptate. All these features are typical of the Hypocreales.

The genus *Calonectria* was established by de NOTARIS (1867) to accomodate *C. daldiniana* de Not., a fungus collected on *Magnolia grandiflora* at Lo-

carno in Italy. Although the type of this fungus was not available to us for study, recently, ROSSMAN (1977) could locate the type material deposited in Rome. According to ROSSMAN, *C. daldiniana* is a later synonym of *C. pyrochroa* (Desm.) Sacc. She has circumscribed the genus to include those nectrioid species with an ascocarp wall structure like that of *C. pyrochroa* and a *Cylindrocladium* anamorph.

The developmental morphology of the type species has not been studied so far. Results of our study on the developmental morphology of *Calonectria rigidiuscula* presented here, for the first time, is the first among the species of the genus *Calonectria*.

BIBLIOGRAPHY

BERKELEY M.J. & BROOME C.E., 1873 – Fungi of Ceylon. *J. Linn. Soc. (Bot.) Lond.* 14 (74) : 116.

BOOTH C., 1960 – Studies of Pyrenomycetes. V. Nomenclature of some Fusaria in relation to their nectroid perithecial states. *Mycol. Pap.* 74 : 1-16.

BOOTH C., 1971 – The genus *Fusarium*. Commonwealth Mycological Institute, Ed. Kew, 237 p.

BRUNT A.A.F. & WHARTON A.L., 1962 Ecology of a gall disease of cocoa in Ghana caused by *Calonectria rigidiuscula* (Berk. & Br.) Sacc. *Ann. appl. Biol.* 50 : 283-289.

FORD E.J., BOURRET J.A. & SNYDER W.C., 1967 – Biological specialization in *Calonectria rigidiuscula (F. decemcellulare)* in relation to green point gall of cocoa. *Phytopathology* 57 : 710-712.

LUTTRELL E.S., 1951 - Taxonomy of the Pyrenomycetes. *Univ. Mo. Stud.* 24 : 1-120.

de NOTARIS G., 1867 - Nuove reclute per la pirenomicetologia Italica. *Recl. Comm. critt. Ital.* 2 : 477-492.

PETCH T., 1916 -- Revisions of Ceylon fungi. *Ann. R. bot. Gdns.* Peradeniya 4 : 153-183.

REICHLE R.E. & SNYDER W.C., 1964 – Heterothallism and ascospore number in *Calonectria rigidiuscula*. *Phytopathology* 54 : 1297-1299.

ROSSMAN A.Y., 1977 – The genus *Ophionectria* (Euascomycetes, Hypocreales). *Mycologia* 69 : 355-391.

SACCARDO P.A., 1878 – Enumeratio Pyrenomycetum Hypocreacearum hucusque cognitorum systemate carpologico dispositorum. *Michelia* 1 : 277-325.

SUBBA RAO M.K., 1938 – Report of the Mycologist, 1936-37. Admin. Rep. Tea Sci. Dep. United Plant. Ass. S. India, pp. 28-37.

SUBRAMANIAN C.V. & BHAT J., 1978 – Developmental morphology of Ascomycetes. III. *Monographella nivalis*. *Rev. mycol.* 42 : 293-305.

WOLLENWEBER H.W., 1926 – Pyrenomyceten-Studien II. *Angew. Bot.* 8 : 168-212.

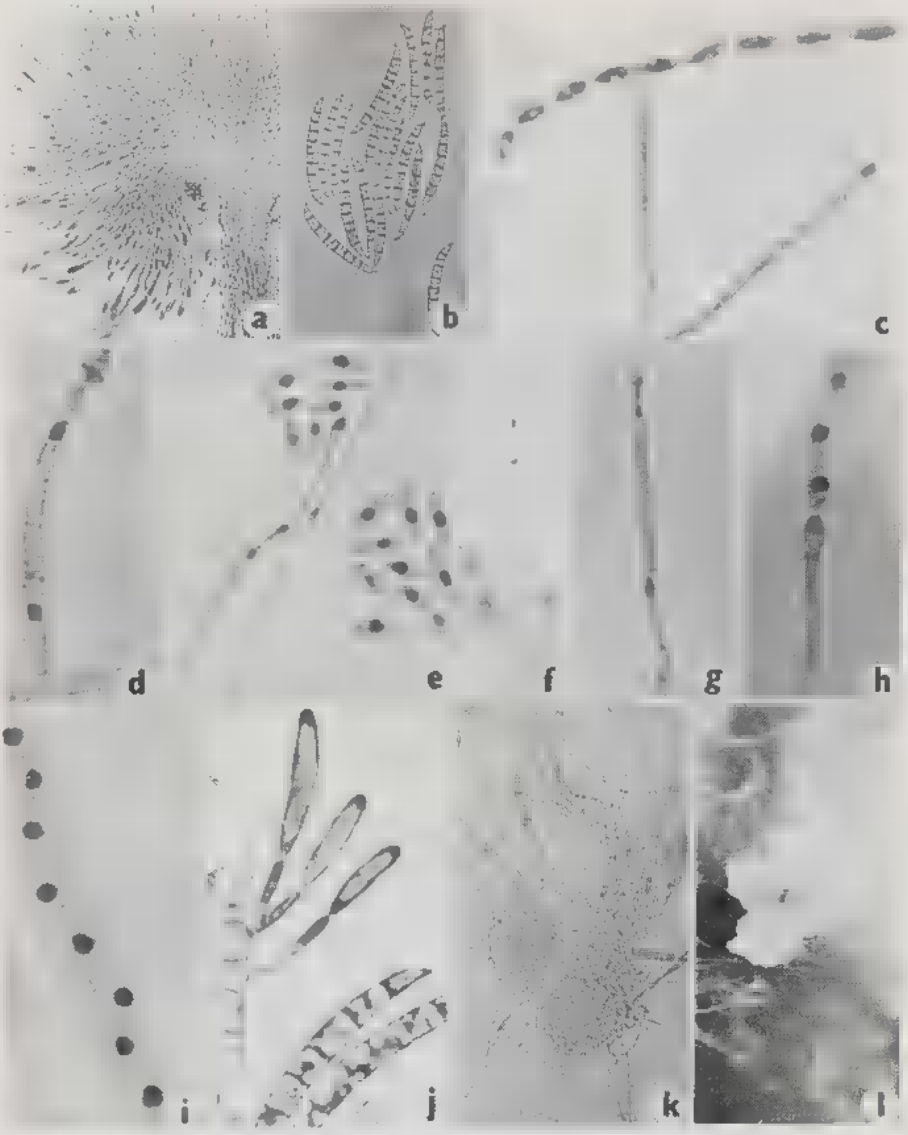


Plate I : *Calonectria rigidiuscula*. – a, young sporodochium with conidiophores, phialides and conidia; b, macroconidia c, phialides : one with a long pseudochain of microconidia; d, phialide showing a cupulate collarette; e, phialide showing a dividing nucleus and a few uninucleate microconidia; f, g, phialides showing one of the daughter nuclei in each migrating into developing conidium; h, a pseudochain of microconidia; i, portion of a macroconidium showing uninucleate cells; j, germ tubes functioning as phialides and producing macroconidia; k, perithecial initials; l, perithecia on twigs in Roux tubes.

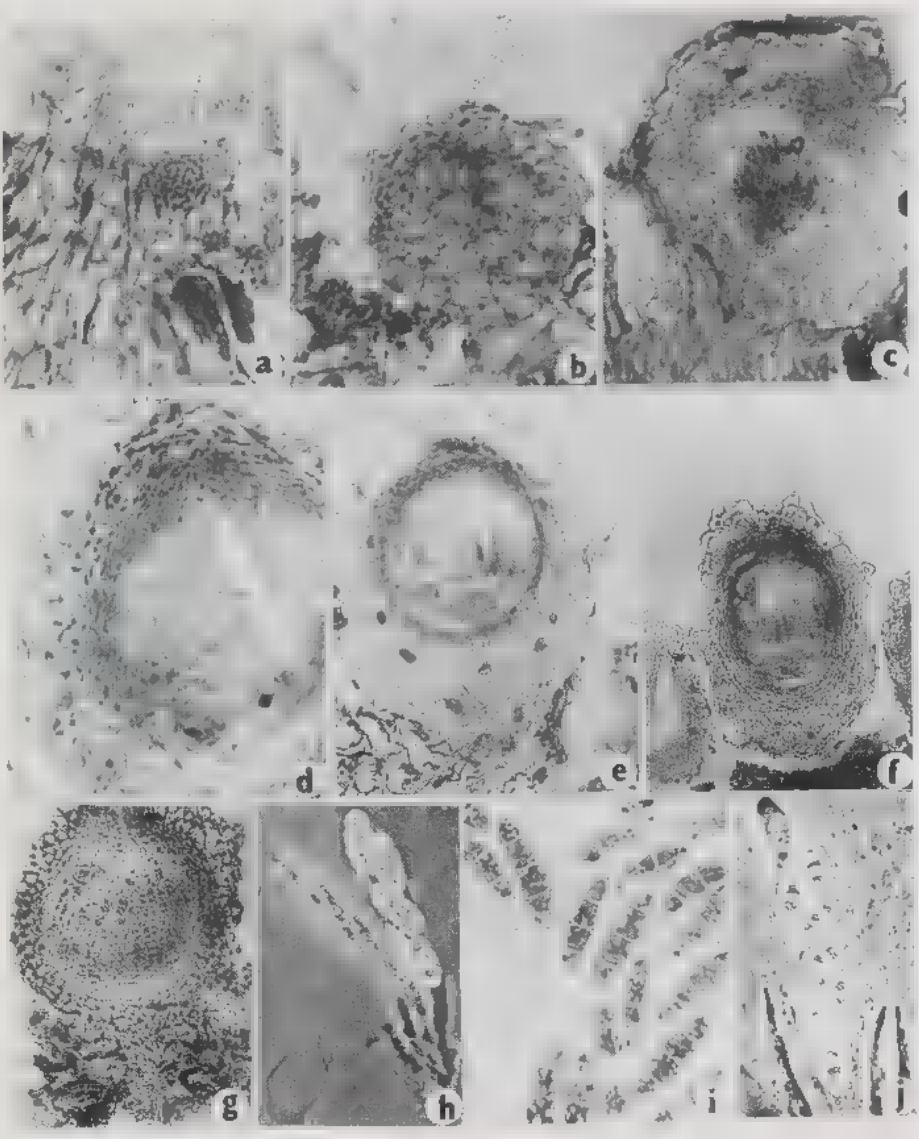


Plate II : *Calonectria rigidiuscula*. – a, portion of a section of a mature stroma showing coiled ascogonium; b-f, longitudinal sections of perithecial centrum showing stages in the development of perithecium. Note in c and d centrum consisting of apical paraphyses; in e and f asci developing interspersed with apical paraphyses; g, section of a mature perithecium; h, 4-spored asci: i, ascospores; j, 8-spored asci.